RNAi Triggered by Symmetrically Transcribed Transgenes in *Drosophila melanogaster*

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ABSTRACT

Specific silencing of target genes can be induced in a variety of organisms by providing homologous double-stranded RNA molecules. *In vivo*, these molecules can be generated either by transcription of sequences having an inverted-repeat (IR) configuration or by simultaneous transcription of sense-antisense strands. Since IR constructs are difficult to prepare and can stimulate genomic rearrangements, we investigated the silencing potential of symmetrically transcribed sequences. We report that Drosophila transgenes whose sense-antisense transcription was driven by two convergent arrays of Gal4-dependent *UAS* sequences can induce specific, dominant, and heritable repression of target genes. This effect is not dependent on a mechanism based on homology-dependent DNA/DNA interactions, but is directly triggered by transcriptional activation and is accompanied by specific depletion of the endogenous target RNA. Tissue-specific induction of these transgenes restricts the target gene silencing to selected body domains, and spreading phenomena described in other cases of post-transcriptional gene silencing (PTGS) were not observed. In addition to providing an additional tool useful for Drosophila functional genomic analysis, these results add further strength to the view that events of sense-antisense transcription may readily account for some, if not all, PTGS-cosuppression phenomena and can potentially play a relevant role in gene regulation.

METHODS of gene silencing are presently attracting great interest, as they provide valuable approaches to the genome functional analysis. Double-strand RNA (dsRNA) is a powerful signal able to induce gene-specific silencing, a phenomenon known as RNA interference (RNAi). RNAi represents a powerful tool for obtaining targeted disruption of a given gene function, overcoming either the need for mutants or the knowledge of a complete and detailed gene structure. Initially observed in *Caenorhabditis elegans* (FIRE *et al.* 1998; MONTGOMERY *et al.* 1998), this method has been demonstrated to be effective in virtually any organism, from protozoa to plants and animals (reviewed by FIRE 1999; BOSHER and LABOUESSE 2000; HAMMOND *et al.* 2001). Although its overall pathway and its physiological role remain to be fully elucidated, it is well established that RNAi represses gene expression by eliciting specific degradation of the homologous target mRNA and thus represents a mechanism of post-transcriptional gene silencing (PTGS; reviewed by FIRE 1999; SHARP 1999; SHARP and ZAMORE 2000; SIJEN and KOOTER 2000). Some events of cosuppression, a phenomenon in which the introduction of transgenes silences homologous chromosomal loci, similarly require an RNA effector molecule and have thus been classified as PTGS phenomena (reviewed by MONTGOMERY and FIRE 1998; BIRCHLER *et al.* 2000). Links between cosuppression and RNAi have been envisaged at both the genetic and molecular level in both animals and plants (see FAGARD *et al.* 2000; KETTING and PLASTERK 2000), supporting the suggestion that some examples of PTGS-cosuppression may rely on the production of dsRNA molecules via read-through transcription starting from a fortuitous promoter flanking the site of insertion (MONTGOMERY and FIRE 1998). This hypothesis implies that a transgene able to produce dsRNA molecules by means of sense-antisense transcription might be able to trigger the silencing of the endogenous homologous loci. We have investigated this possibility, with the aim of establishing whether this type of transgene could provide an additional tool useful for functional genetic studies. In fact, although RNAi can rapidly and simply be induced by injections of specific dsRNA into living organisms, this experimental approach has a number of disadvantages, given that the interference with gene expression may be transient and genes expressed in later stages of development cannot be inactivated (KENNERDELL and CARTHEW 1998; MONTGOMERY *et al.* 1998; MISQUITTA and PATERSON 1999; WIANNY and ZERNICKA-GOETZ 2000). In organisms for which transgenic technology is available, these problems have recently been circumvented by using heritable transgenes having an inverted-repeat (IR) configuration, which are able to produce dsRNA molecules *in vivo* as extended hairpin RNA. These transgenes have already been used to generate efficient RNAi

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in several organisms, such as *C. elegans* (Tavernarakis *et al.* 2000), Trypanosoma (Bastin *et al.* 2000; Shi *et al.* 2000), Drosophila (Fortier and Belote 2000; Kennerdell and Carthew 2000; Lam and Thummel 2000; Martinek and Young 2000), and plants (Chuang and Meyerowitz 2000; Smith *et al.* 2000). However, some IR constructs may be difficult, if not impossible, to clone. Moreover, genomic IR sequences are mitotically unstable and stimulate rearrangements in eukaryotic cells (Leach 1994; Bi and Liu 1996; Lobachev *et al.* 2000), raising the possibility that IR transgenic lines may be characterized by genetic instability. High rates of structural instability have in fact been reported for palindromic transgenes in mice, where mitotic mosaicism and rearranged versions of the transgene have been detected in the progeny (Collick *et al.* 1996; Akgun *et al.* 1997; Lewis *et al.* 1999; Waldman *et al.* 1999). To avoid these problems, we have investigated the silencing potential of constructs designed to generate Gal4-dependent simultaneous transcription of both sense-antisense strands. We report here that, in Drosophila, these transgenes are capable of repressing gene activity *in vivo*, in the transformed adult flies. According to the expression pattern of the *Gal4* line used as driver, RNAi can be induced ubiquitously, or in selected tissues at specific developmental times, and the silencing effect is not significantly spread to the neighboring cells. In addition, to extend the potential of RNAi's applications, our results add further strength to the view that spontaneously occurring transcription of sense-antisense strands can potentially play a relevant role in the regulation of gene expression and support the hypothesis that unintended symmetrical transcription causing aberrant dsRNA formation may readily account for some, if not all, PTGS-cosuppression phenomena.

MATERIALS AND METHODS

Plasmid construction: To prepare the *Sym-pUAST-w* construct, the Drosophila transformation *pUAST* vector (Brand and Perrimon 1993) was modified by sequential insertion of three genetic cassettes. In a first cloning step, we duplicated the SV40 polyadenylation region, by inserting a *pUAST*-derived 871-bp *Bam*HI-SV40-*Bam*HI polyadenylation cassette into the *Bam*HI site of the partially digested *pUAST* vector, upstream of the *UAS* array, and with opposite orientation with respect to the SV40 polyadenylation sequence originally present in the vector. In a second step, we duplicated—with opposite orientation—the five-copy array of the *UAS*-activating sequence, by directionally inserting a *pUAST*-derived 364-bp *Xba*I-*UAS-Xho*I cassette into the unique *Xba*I/*Xho*I sites of the previously obtained construct. After the insertion of these two genetic cassettes, the *pUAST* cloning polylinker carried on both sides two identical but oppositely oriented regulatory regions, each composed of a five-copy array of the *UAS*-activating sequence coupled with an inversely oriented SV40 polyadenylation site. In a third step, a 1.4-kb *Eco*RI-*white-Eco*RI cassette, containing an exon-rich genomic fragment of the Drosophila *white* (*w*) gene, was ligated into the unique *Eco*RI site between the two inverted *UAS*-SV40 sequences. The *Bam*HI-SV40-*Bam*HI, *Xba*I-*UAS-Xho*I, and *Eco*RI-*w-Eco*RI (from nucleotides 10886 to 12222 of GenBank accession no. X02974) cassettes represent PCR-amplified fragments obtained using, respectively, the following pairs of primers: *Bam*HI-5′SV40 (5′-TACGGATCCGATCT TTGTGAAGGAACC-3′) and *Bam*HI-3′SV40 (5′-ATCGGATCCA CTAAGGCCTTCTAGTGG-3′); *Xba*I-5′*UAS* (5′-TATCTAGACT TGCATGCCTGCAGGTC-3′) and *Xho*I-3′*UAS* (5′-TACTCGAG AGTTCTCTTCTTGTATTC-3′); *Eco*RI-5′*w* (5′-TCTGAATTCG CAGAGCTGCATTAAC-3′) and *Eco*RI-3′*w* (5′-ATTGAATTCC TTGAGCACCGACAG-3′). All PCR-amplified fragments were cloned in *pBluescript* or *pUC18* in appropriate orientation for subsequent manipulations. To obtain the *pUAST-IR-w* and *pUAST-IR$_{\varphi}$-w* constructs, inverted repeats of the *w* cassette were initially generated in *pBluescript* and then cloned in the *pUAST* vector according to the following steps. First, a *pBluescript* plasmid containing the 1.4-kb *Eco*RI-*w-Eco*RI cassette (*pBS1.4w* clone) was digested with *Bam*HI and *Hin*dIII and the resulting *w* fragment was cloned into the corresponding restriction sites of *pUC18* vector, to give the *pUC1.4w* clone. This clone was digested with *Kpn*I and *Hin*dIII, and the 1.4-kb *w* resulting fragment was inserted into the corresponding sites of the *pBS1.4w* clone, obtaining a construct with two copies of the 1.4-kb *w* fragment arranged as tail-to-tail repeats (*pBS-IRw* clone). A second construct (*pBS-IR$_{\varphi}$w* clone) was subsequently generated by inserting a 200-bp PCR-amplified fragment of the *Escherichia coli lacZ* gene into the *Eco*RV restriction site of the *pBS-IRw* clone. The *lacZ* DNA spacer was amplified using the primers *Eco*RV-*lacZ*-5′ (5′-ATTGATATCTGTATGAACGGTCTGGTC-3′) and *Eco*RV-*lacZ*-3′ (5′-ATTGATATCCAGCGCCACCATCCAG TGC-3′). Finally, the *pUAST-IR-w* and *pUAST-IR$_{\varphi}$-w* transgenes were generated by individually inserting the spaced or perfect *w* IRs (from the *pBS-IR$_{\varphi}$w* and the *pBS-IRw* clone, respectively) as *Kpn*I/*Xba*I fragments into the corresponding restriction sites of the *pUAST* vector. The transformation constructs used to transcribe *in vivo* only the sense (*pUAST-w$_s$*) or the antisense strand (*pUAST-w$_{as}$*) of the *w* cassette were obtained by inserting the 1.4-kb *Eco*RI-*w-Eco*RI DNA fragment into the unique *Eco*RI site of the *pUAST* vector and subsequently recovering recombinant clones in both orientations.

The *Sym-pUAST-mfl* construct was obtained by substituting the 1.4-kb *Eco*RI-*w-Eco*RI cassette of the *Sym-pUAST-w* transgene with a 1.7-kb PCR-generated *Eco*RI-*minifly-Eco*RI cassette. This fragment, corresponding to nucleotides +619 to +2327 of the *mfl* gene (GenBank accession no. AF097634), was obtained by using as primers the *Eco*RI-5′*mfl* (5′-ATGAATTCAAGTACG CAAGGAGAAGA-3′) and *Eco*RI-3′*mfl* (5′-TAGAATTCGTGTT TTTCATTGGG-3′) oligonucleotides. Structure of each cloned construct was verified by a combination of PCR amplification, restriction mapping, and DNA sequencing. In all the experiments involving construction of clones containing inverted repeats, the *E. coli* SURE strain (Stratagene, La Jolla, CA) was used as a bacterial host capable of tolerating IRs; the DH5α strain was utilized in the other cases.

Drosophila strains and *P*-mediated transformation: The genetic markers and chromosomes used in our experiments are described in Lindsley and Zimm (1992); most of the stocks were from Bloomington Drosophila Stock Center. In the *Gal4* expression-defective *Act5C-y$^+$-Gal4* strain, the P(*Act5C-y$^+$-Gal4*) transposon was inserted on the second chromosome and carried a functional copy of the *yellow* (*y*) gene, flanked by two *FRT* target sites of the *FLP*-recombinase, between the *Actin5C* promoter and the *Gal4* gene (Ito *et al.* 1997). The Gal4-producing *Act5C-Gal4* strain was obtained from the *Act5C-y$^+$-Gal4* stock by *in vivo FLP*-mediated excision of the *y* gene (Ito *et al.* 1997). *P*-element-mediated germline transformation and insertion mapping were performed as previously described (Giordano *et al.* 1999). In all Drosophila transgenic lines, integrity and *in vivo* stability of the IR-containing constructs were verified by Southern blot experiments.

Cloning techniques, DNA and RNA analysis, and *in situ* hybridization: Basic cloning techniques, DNA and RNA extraction, PCR amplifications, labeling, and sequencing techniques were carried out essentially according to SAMBROOK *et al.* (1989). For Southern blot genomic analysis, 5 μg of *Bam*HI-digested DNA was electrophoresed, transferred to Hybond-NX (Amersham, Arlington Heights, IL) filters, and hybridized using the 1.4-kb *w* cassette as probe. For each transgenic line, four DNA samples, each derived from a pool of 30 adult flies, were examined. For Northern blot analysis, 3 μg of poly(A)$^+$ or 5 μg of total RNA were electrophoresed and transferred to Hybond-NX (Amersham) filters for hybridization. Single-stranded probes spanning *w* exons 2–4 were generated by asymmetrical PCR reactions utilizing forward or reverse M13 primers and the *pBS1.4w* clone as template. Endogenous *w* mRNA was analyzed by poly(A)$^+$ Northern blot experiments using as probe a *w* genomic fragment external to the 1.4-kb *w* cassette, spanning the *w* exons 5 and 6 (from coordinates 12490 to 13230 of GenBank accession no. X02974). *In situ* hybridizations were performed as previously described (GIORDANO *et al.* 1999). RNA quantitative analyses were carried out with ImageQuaNT software and the Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

Eye pigment determination: Eye pigment determination assays were performed on the F_1 heterozygous females of straight-wing phenotype, derived, respectively, from the crosses between w^+; *P(Act5C-Gal4)/CyO* or w^+; *P(Act5C-y$^+$-Gal4)/CyO* virgin females and males of generic w^{1118}; *P(UAS)/CyO* genotype, where the *P(UAS)* symbol indicates the appropriate *UAS* construct, as described in Figure 3B. To quantify the eye pigment, virgin females of the appropriate phenotypic class were collected, aged for 5 days after eclosion, and frozen in liquid nitrogen. Heads were manually dissected and pooled, and the pigments were extracted according to ASHBURNER (1989b). For each genotypic class, the extraction was performed from 5 heads if flies displayed red eyes or from 15 heads if eyes were orange or light yellow. Pigment absorption was measured at 450 nm; five extractions were performed for each genotypic class, and the mean values of the absorption per head were calculated.

RESULTS AND DISCUSSION

Construction and effectiveness of the *Sym-pUAST-w* transgene: To investigate the potential of symmetrical transcription in producing RNAi we constructed Drosophila heritable transgenes able to generate dsRNA molecules by simultaneous transcription of both strands. To take advantage of the yeast *Gal4/UAS* binary system to modulate the expression of these transgenes, we modified (see MATERIALS AND METHODS) the previously described *pUAST* vector (BRAND and PERRIMON 1993) in a way that its cloning polylinker was flanked on both sides by two identical but oppositely oriented regulatory regions, each composed of a five-copy array of the *UAS* activating sequence coupled with an inversely oriented SV40 polyadenylation site. The obtained construct was thus able to drive simultaneous transcription of both sense-antisense strands of a given DNA insert from two convergent arrays of the Gal4-responsive *UAS* regulatory elements. The silencing potential of this type of construct was first tested by preparing *Sym-pUAST-w* (symmetrically transcribed *pUAST-w*), a transgene containing a 1.4-kb genomic segment spanning exons 2–4 of the Drosophila *white* (*w*) gene. This transgene, whose structure is depicted in Figure 1A, would symmetrically transcribe a genomic segment of the *w* gene, reproducing an event of read-through transcription whose occurrence has been invoked as a possible cause of PTGS-cosuppression events (MONTGOMERY and FIRE 1998). The *w* gene was chosen because the *UAS*- and *Gal4*-bearing transposons both included a copy of the *w* coding sequence (Figure 1A), usually referred to as mini-*white* (mini-*w*), whose expression level can easily be followed phenotypically, and can thus provide an ideal target for the silencing effect. mini-*w* elements that are transcribed from their own promoter are in fact routinely utilized as selectable gratuitous transformation markers (see ASHBURNER 1989a), since in a *w* mutant background their expression represents the unique source of the eye pigmentation and directly establishes the eye color of the fly. In a first set of experiments, we thus utilized strains having a w^{1118} (a *white* partial deletion) genotype, so that their eye pigmentation was entirely dependent on the expression of the mini-*w* reporters. mini-*w* elements are known to generate different eye-color phenotypes depending on the specific transposon's insertion site in the genome. In our experiments, we obtained three independent transformed lines carrying a single-copy insertion of the *Sym-pUAST-w* transgene on the second chromosome; as a consequence of the position effect, all had faintly pigmented eyes. These lines were then mated to the *Act5C-Gal4* strain, which expresses the *Gal4* activator gene under the control of the constitutive *Actin5C* promoter (ITO *et al.* 1997), to activate ubiquitously the symmetrical transcription of the transgene in the F_1 progeny. In this cross, the two parental strains had markedly different eye-color phenotypes, that is, pale yellow for *Sym-pUAST-w* and orange for *Act5C-Gal4* (see Figure 1B). Given that the simultaneous presence of two independent mini-*w* elements is known to have additive effects on eye color (LEVIS *et al.* 1985), the F_1 progeny were expected to have more intense eye pigmentation than each parental, unless the transcriptional activity of the *Sym-pUAST-w* transgene was able to silence the expression of the mini-*w* reporters. We thus analyzed the F_1 progeny, comparing for the eye-color phenotype the *Act5C-Gal4/Sym-pUAST-w trans*-heterozygous females to their daughters of *Sym-pUAST-w/CyO* and *Act5C-Gal4/CyO* genotypes, which carried, respectively, only one of the two parental transposons. Only the F_1 females were carefully compared, to avoid any effect related to dosage compensation of the mini-*w* elements. Interestingly, phenotypic analysis showed that all the *Act5C-Gal4/Sym-pUAST-w* F_1 females had light yellow eyes (see Figure 1B) and thus exhibited a strong silencing of the mini-*w* expression. No additional or abnormal phenotype was observed in these silenced flies, suggesting that the silencing was sequence specific. The effect was also stably maintained during the entire adult life and identically reproduced in hybrids having

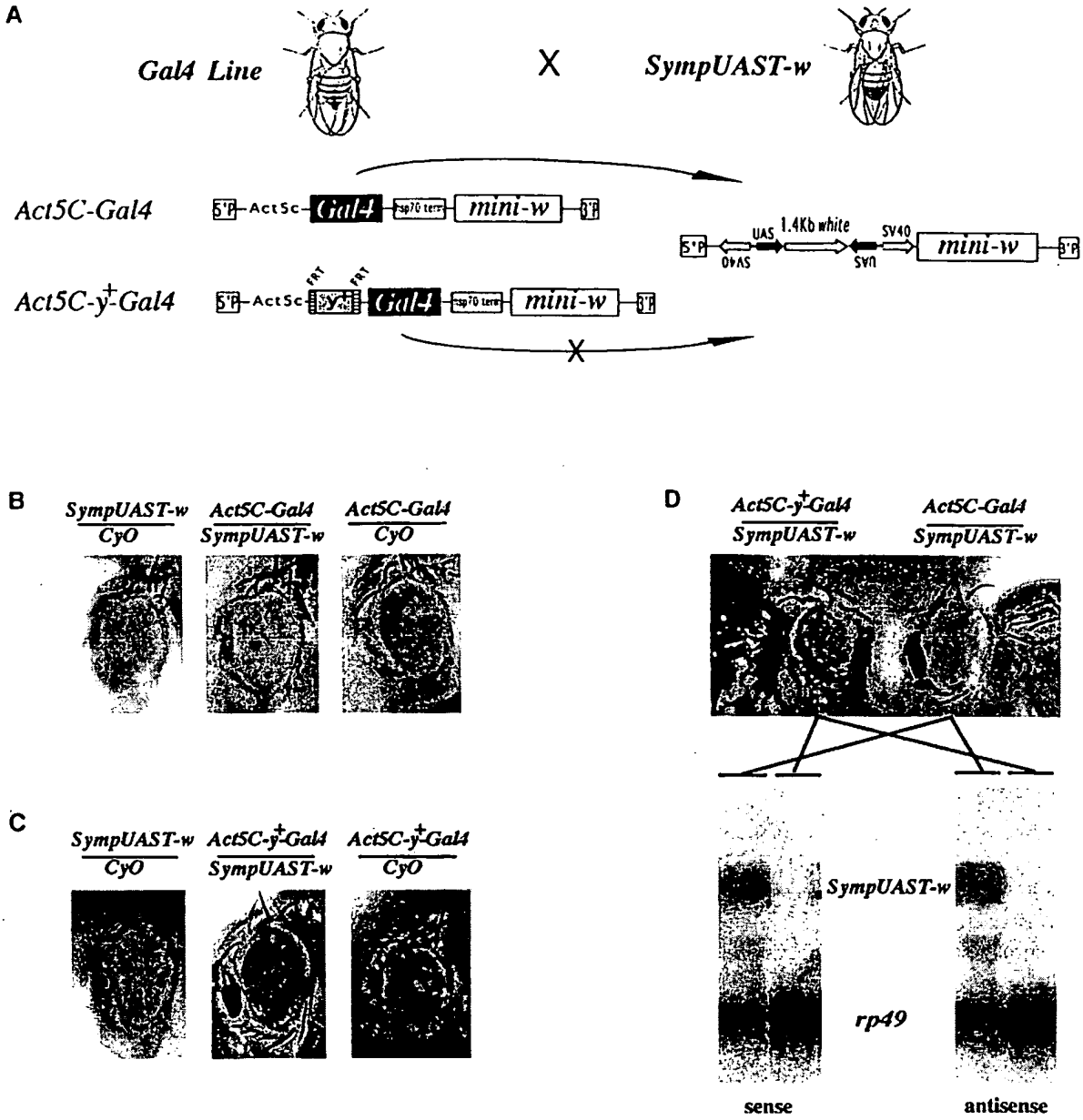

FIGURE 1.—Transcriptional activation of the *Sym-pUAST-w* transgene triggers phenotypic silencing of the mini-*w* reporters. (A) Diagram of the genetic crosses performed to test the silencing potential of the *Sym-pUAST-w* transgene, whose schematic organization is depicted on the right. *Sym-pUAST-w* transgenic lines (on the right) were mated to the *Act5C-Gal4* strain, which expresses the *Gal4* activator gene under the control of the constitutive *Actin5C* promoter, to induce ubiquitous symmetrical transcription of the transgene in the F_1 progeny. Crossing to the *Act5C-y*$^+$*-Gal4* line, which carries a *Gal4*-defective transposon, was used as a negative control. As shown in the scheme, the *Sym-pUAST* and the Gal4-producing or nonproducing transposons all carry a copy of the mini-*w* gene, whose expression can easily be followed at the phenotypic level in a *w* mutant background. (B) The transcriptional activation of the *Sym-pUAST-w* transgene in the *Sym-pUAST-w/Act5C-Gal4* F_1 *trans*-heterozygous females (middle) causes phenotypic silencing of the mini-*w* reporters. Phenotypes of daughters of *Sym-pUAST-w/CyO* and *Act5C-Gal4/CyO* genotypes, respectively, are shown at left and right. (C) Absence of silencing is instead observed in *Sym-pUAST-w/Act5C-y*$^+$*-Gal4 trans*-heterozygous females (middle), in which the transcription of the transgene is not activated. In fact, phenotypic comparison of these individuals with their daughters of *Sym-pUAST-w/CyO* and *Act5C-y+-Gal4/CyO* genotypes (left and right) indicates that they exhibit the expected additive effect on the eye pigmentation. (D) Northern blot analyses of total RNA preparations confirm that sense and antisense strands of the *w* cassette are both actively transcribed in the silenced (*Sym-pUAST-w/Act5C-Gal4*), but not in the unsilenced *trans*-heterozygotes (*Sym-pUAST-w/Act5C-y*$^+$*-Gal4*). A probe corresponding to the gene encoding the Drosophila rp49 protein was also utilized to control the amount of RNA (5 μg) loaded in each lane.

different genomic insertions of the *Sym-pUAST-w* transgene.

***Sym-pUAST-w*-mediated gene silencing is dependent on Gal4 transcriptional activation:** A crucial point was that of establishing whether the silencing effect exerted by *Sym-pUAST-w* was induced by homology-dependent

DNA/DNA interactions (reviewed by SELKER 1999; BIRCHLER *et al.* 2000) or instead was directly dependent on the transcriptional activation of the transgene. To assess this point, we crossed the *Sym-pUAST-w* lines to *Act5C-y*$^+$*-Gal4* flies, which carry a *Gal4*-defective transposon. The *Act5C-y*$^+$*-Gal4/Sym-pUAST-w* heterozygous females provided an ideal control of the eye-color phenotype in the absence of *Sym-pUAST-w* transcriptional activation, since the *Act5C-Gal4* line was originally obtained from the *Act5C-y*$^+$*-Gal4* strain after *FRT/FLP*-mediated excision of y^+ sequences (ITO *et al.* 1997). This excision event rescued *Gal4* function without altering the level of expression of the transposon's mini-*w* reporter, so that the *Act5C-y*$^+$*-Gal4* and *Act5C-Gal4* flies exhibited identical eye-color phenotype (see Figure 1, B and C). Strikingly, *Sym-pUAST-w/Act5C-y*$^+$*-Gal4 trans*-heterozygotes all showed an additive effect on the eye pigmentation, as expected in the absence of any silencing effect (see Figure 1C). This observation indicated that the transcriptional activation of the *Sym-pUAST-w* transgene was actually responsible for the mini-*w* silencing. Northern blot analysis of total RNA preparations confirmed that the transgene was actively expressed in the silenced flies, where both sense and anti-sense transcripts accumulated at high levels, whereas it was completely inactive in the *Sym-pUAST-w/Act5C-y*$^+$*-Gal4* controls (Figure 1D). Finally, when the *Act5C-Gal4* element was combined with transgenes transcribing only the sense (*pUAST-w*$_s$ construct) or the antisense (*pUAST-w*$_{as}$ construct) strand of the 1.4-kb *w* insert, no appreciable silencing was observed (data not shown; see below, Figure 3B). Hence, all the data are compatible with the view that the mini-*w* phenotypic silencing is triggered by the active synthesis of *w* dsRNA molecules, which might possibly be recruited by the RNA interference pathway.

To test whether the *Sym-pUAST*-induced RNAi could appropriately be modulated by means of the *Gal4/UAS* system, we analyzed the phenotype of the progeny generated by crossing the *Sym-pUAST-w* lines to strains expressing *Gal4* under the control of various promoters. In these crosses we also wished to check whether the *Sym-pUAST-w* transgene could effectively disrupt the expression of a *wild-type* genomic copy of the *w* gene, so we kept the *Sym-pUAST-w* transgenic lines in w^+/w^+ genetic background, to generate a female progeny having a w/w^+ heterozygous genotype. Despite the increased dosage of the target, we observed a dramatic reduction of the eye pigment level in *Sym-pUAST-w/β-tub-Gal4 trans*-heterozygotes, which expressed the *Gal4* gene under the control of the strong ubiquitous *β-tubulin* promoter (see Figure 2). Two strains in which *Gal4* expression was restricted to specific cell types were also tested as drivers. In these strains, *Gal4* expression was under the control of the *elav* or the *sevenless* (*sev*) promoter, respectively. The *elav* promoter, which directs persistent *Gal4* expression in ommatidial photoreceptor cells (LIN and GOODMAN 1994), triggered an appreciable silencing of the *w* gene in the *Sym-pUAST-w/elav-Gal4* heterozygotes. In contrast, the *sev* promoter, which drives transient *Gal4* expression in a subpopulation of ommatidial precursor cells (BRUNNER *et al.* 1994), was unable to induce a detectable reduction of the *wild-type* red color. However, we noted that *Sym-pUAST-w/sev-Gal4* heterozygotes had red/brown-mottled eyes (Figure 2; see inset), a phenotype highly reminiscent of that of individuals showing position-effect variegation at the *w* locus (BIRCHLER *et al.* 1994), whose eyes are characterized by small flecks of red- and brown-pigmented ommatidia. This variegated phenotype is expected if *w* silencing occurred in a restricted population of photoreceptor progenitor cells and thus fits nicely with the pattern of *Gal4* expression in these heterozygous flies. Moreover, it reveals that even a transient expression of the *Sym-pUAST-w* transgene is capable of interfering appreciably with gene expression and thus generates noticeable adult phenotypes. This is consistent with the long-term effects of RNAi observed in several organisms (FIRE 1999). Taken together, the results obtained support the conclusion that symmetrically transcribed transgenes can effectively be utilized to block the expression of endogenous genes and may thus represent a useful tool for the functional analysis of the Drosophila genome.

FIGURE 2.—Gal4-dependent silencing of the endogenous *w* gene. Phenotypes of w^+/w heterozygous females in which the *Sym-pUAST-w* transgene was either inactive (left) or activated by *β-tub-Gal4*, *sev-Gal4*, or *elav-Gal4* drivers, respectively, are shown. According to the promoter's strength, a strong silencing is observed in *Sym-pUAST-w/tub-Gal4* heterozygotes, whereas *Sym-pUAST-w/elav-Gal4* individuals exhibited only a weak disruption of *w* expression. The variegated phenotype observed in *Sym-pUAST-w/sev-Gal4* flies (inset) is in good agreement with the restricted expression pattern imposed on the *Gal4* gene by the *sev* promoter.

Phenotypic comparison of flies silenced by IR or symmetrically transcribed *w* transgenes: A method to express dsRNA molecules as extended hairpin RNA has recently been reported to be capable of generating efficient RNAi in Drosophila (FORTIER and BELOTE 2000; KENNERDELL and CARTHEW 2000; LAM and THUMMEL 2000; MARTINEK and YOUNG 2000; PICCIN *et al.* 2001). According to this method, dsRNA can be produced from

transgenes exhibiting dyad symmetry. However, this approach is complicated by the fact that IR constructs may be difficult to clone in *E. coli* (COLLINS 1981). Also, in eukaryotes, from yeast to mammals, IR sequences are unstable and undergo genetic rearrangements (see LEACH 1994; WALDMAN *et al.* 1999; LOBACHEV *et al.* 2000). In mice, palindromic transgenes show high rates of structural instability, which is associated with the presence of an inverted repeat within each transgene (COLLICK *et al.* 1996; AKGUN *et al.* 1997; LEWIS *et al.* 1999; WALDMAN *et al.* 1999). Rearranged versions of IR transgenes are generated at high frequency, and this aspect might complicate the analysis of knock-out or knock-down experiments. Symmetrically transcribed transgenes are instead expected to be prepared more easily and maintained more stably and can thus provide a convenient alternative approach, if it is established that they can induce RNAi at a comparable efficiency. With the aim of carrying out a quantitative comparison between the silencing effect induced by IR and symmetrically transcribed transgenes, we prepared two types of IR *w* constructs. In a first type, named *pUAST-IR$_{sp}$-w*, two inverted repeated copies of the same 1.4-kb *w* segment carried by the *Sym-pUAST-w* transgene were inserted downstream of the *UAS* regulatory elements, with 200 bp spacing. In a second type, named *pUAST-IR-w*, the same IRs were inserted without spacing, to produce a perfect snap-back RNA (Figure 3A; see MATERIALS AND METHODS).

Several independent lines carrying a single-copy insertion of each IR construct on the second chromosome were then obtained. Among them, we selected for each type of IR transgene two lines having an eye-color phenotype similar to that displayed by those carrying *Sym-pUAST-w*, to facilitate the phenotypic comparison of F_1 flies resulting by individually combining each transgene with the Gal4-producing or nonproducing transposons. To enhance the sensitivity of the assays, the phenotypic and quantitative analyses were again performed on F_1 females having the w/w^+ heterozygous genotype, who thus carried, in addition to the two mini-*w* transposons, a *wild-type* genomic copy of the *w* gene. When in combination with the *Act5C-Gal4* active element, the *Sym-pUAST-w*, *pUAST-IR$_{sp}$-w*, and *pUAST-IR-w* transgenes all induced an appreciable reduction of the eye pigmentation compared to that of their *Gal4*-defective sibling controls (Figure 3A, compare Gal4+ and Gal4− lanes in each panel). Flies carrying either of the two types of IR transgenes appeared to be more strongly silenced, although those carrying the *pUAST-IR-w* construct were remarkably heterogeneous at the phenotypic level (Figure 3A, compare top and bottom). Moreover, we noted that a fraction of these flies exhibited a variegated eye-color phenotype, mainly characterized by the presence of darker red patches (Figure 3A, see arrow) on a less pigmented, orange background. Size and position of the patches varied among individuals, suggesting that *w* mosaicism occurred throughout development.

Quantitative estimate of the silencing effect induced by IR or symmetrically transcribed *w* transgenes: To estimate the silencing efficiency of each type of transgene, we determined the eye pigment content in the three types of silenced and control hybrid females. Although the eye color was only slightly reduced in the *Sym-pUAST-w* transgenic lines, the red pigment content was reduced to ~10% with respect to the controls (Figure 3B). This discrepancy is apparent only because, as reported in the literature, variation in the eye pigment level cannot be easily appreciated visually (ZIEGLER-GUNDER and HADORN 1958; GREEN 1959; MACKENZIE *et al.* 1999). The pigment amount was reduced even further in flies homozygous for a third chromosome *Sym-pUAST-w* insertion (Figure 3B, line 7b), indicating that the silencing effect is reinforced by increasing the transgene dosage. In contrast, the transgenes transcribing only the sense (*pUAST-w$_s$* construct) or the antisense (*pUAST-w$_{as}$* construct) strand did not induce any significant reduction of the pigment amount, consistent with the view that the genetic silencing is dependent on the production of dsRNA. As already indicated by the phenotypic analysis, the eye pigment content was more drastically reduced in the *pUAST-IR$_{sp}$-w* and *pUAST-IR-w* silenced flies, where it was found, respectively, to be ~2 and 3% of the controls. We then checked, by Northern blot analysis, whether the gene silencing triggered by the *Sym-pUAST-w* transgene was paralleled by depletion of the homologous target mRNA. To detect specifically the endogenous *w* mRNA, we used a probe derived from *w* mRNA sequences not included in the *SympUAST-w* transgene. As shown in Figure 3C, the level of the endogenous *w* mRNA was reduced to ~24% of the controls in these silenced flies and, more drastically, to ~9% in those expressing the *pUAST-IR$_{sp}$-w* transgene. Thus, all the results obtained indicate that *Sym-pUAST-w* can actively silence the endogenous copy of the *w* gene, although it is less effective (approximately threefold) than the corresponding IR transgenes. One of the factors limiting the efficiency of the *Sym-pUAST-w* construct might be the collision of polymerases during simultaneous transcription on both strands. However, we noted that *Sym-pUAST-w* sense and antisense transcripts were produced at a quite appreciable level (see Figure 1D). Thus, it is more likely that only a minor fraction of the sense-antisense RNA molecules produced by this transgene anneal to form dsRNA and that this may limit its silencing efficiency. Accordingly, our findings that the *Sym-pUAST-w* effectiveness can be modulated by the strength of the *Gal4* promoter, and reinforced by increasing the transgene dosage, support the hypothesis that the amount of dsRNA molecules produced may be a limiting factor. It is noteworthy that these effects have been reported also for IR transgenes (PICCIN *et al.* 2001), in spite of the fact that a very limited number of dsRNA molecules are sufficient to induce RNAi when injected in Drosophila embryos (KENNERDELL and CARTHEW 1998). It is

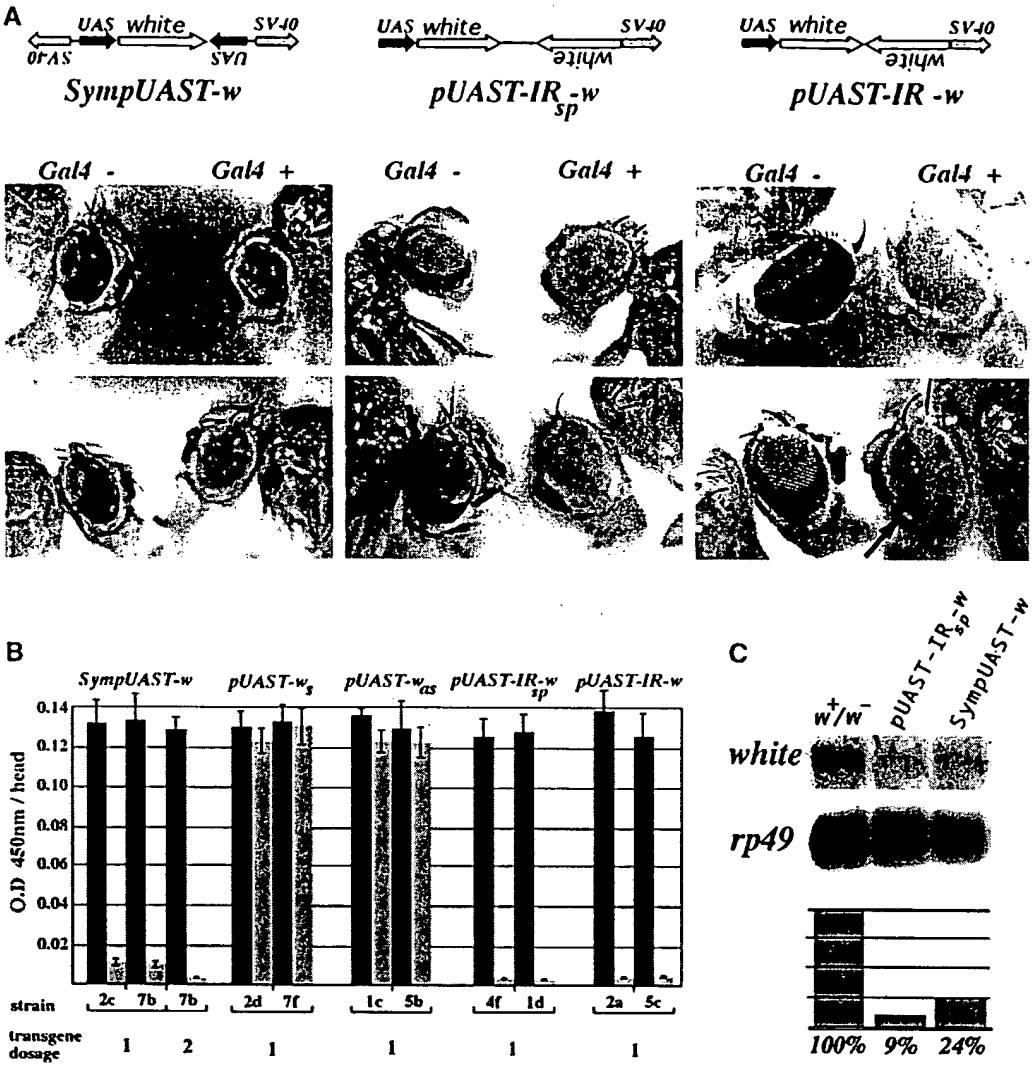

FIGURE 3.—Comparison of the silencing effect induced by symmetrically transcribed or IR *w* transgenes on target gene expression. (A) At the top, schematic structure of three transgenes expressing *w* dsRNA is shown. The *SympUAST-w* transgene produces dsRNA by symmetrical transcription, while the two IR constructs carry the same segment of the *w* gene arranged as a dimer with dyad symmetry, spaced (*pUAST-IR-w$_{sp}$*) or not (*pUAST-IR-w*) by a 200-bp segment of nonpalindromic DNA. Below, phenotypes of w^+/w heterozygous females carrying each type of transgene, either in combination with the *Act5C-y$^+$*-Gal4 inactive transposon (lane Gal4−) or activated by the *Act5C-Gal4* driver (lane Gal4+), are shown. Within each line, the silenced flies carrying the *Sym-pUAST-w* or the *pUAST-IR-w$_{sp}$* transgene showed a substantially uniform phenotype (compare the two individuals of the same genotype shown in the top and bottom), whereas those carrying the *pUAST-IR-w* construct are remarkably heterogeneous at the phenotypic level, with many exhibiting a variegated eye color (see arrow). (B) Quantitative estimate of the eye pigment content accumulated in each of the three types of silenced (shaded bars) and control hybrid females (solid bars). In the transgenic line 7b, which carries a third chromosome insertion of the *Sym-pUAST-w* transgene, the silencing effect has been checked in both heterozygosity (transgene dosage 1) or homozygosity (transgene dosage 2). Note that constructs transcribing only the sense (*pUAST-w$_s$*) or the antisense strand (*pUAST-w$_{as}$*) did not induce any significant reduction of the pigment amount. For each transgene, phenotypic and quantitative analyses were performed in at least two independent transformed strains. (C) Northern blot analysis of poly(A)$^+$ RNA preparations obtained from *pUAST-IR-w$_{sp}$* or *Sym-pUAST-w* silenced flies. To detect specifically the level of the endogenous *w* mRNA, the probe used was derived from *w* mRNA sequences not included in the *Sym-pUAST-w* transgene (see MATERIALS AND METHODS). In both types of silenced flies, the level of endogenous *w* mRNA was strongly reduced with respect to the controls (w^+/w). A probe corresponding to the gene encoding the Drosophila rp49 protein was also utilized to control the amount of RNA loaded in each lane.

therefore possible that both types of transgenes produce, *in vivo*, a significantly lower level of active dsRNA species than expected on the basis of their transcriptional activity.

Structural stability of IR or symmetrically transcribed *w* transgenes: Flies with variegated eyes were detected in both the *pUAST-IR-w* lines examined, indicating that this construct can generate mitotic mosaicism. We excluded the possibility that the eye mosaicism could be caused by a position effect, as *w* mosaics were present in both transgenic lines. In addition, it has recently been shown that heterochromatin-mediated transcriptional silencing of a Gal4-dependent promoter is efficiently counteracted by Gal4 binding (AHMAD and HENIKOFF 2001). To check whether *w* variegation was due to structural instability of the *pUAST-IR-w* palindromic construct, we examined the two *pUAST-IR-w* transgenic lines by Southern blot genomic analysis. As a control, we analyzed also flies carrying either the *pUAST-IR$_{sp}$* or the *Sym-pUAST-w* construct. Flies of each genotype were all homozygous for the w^{1118} allele (a *white* partial deletion). Two independent lines were examined for each transgene. For each line we analyzed four *Bam*HI-digested DNA samples, each derived from a pool of 30 adult flies, by hybridization to the 1.4-kb *w* cassette used as probe. This probe should detect three bands, two of

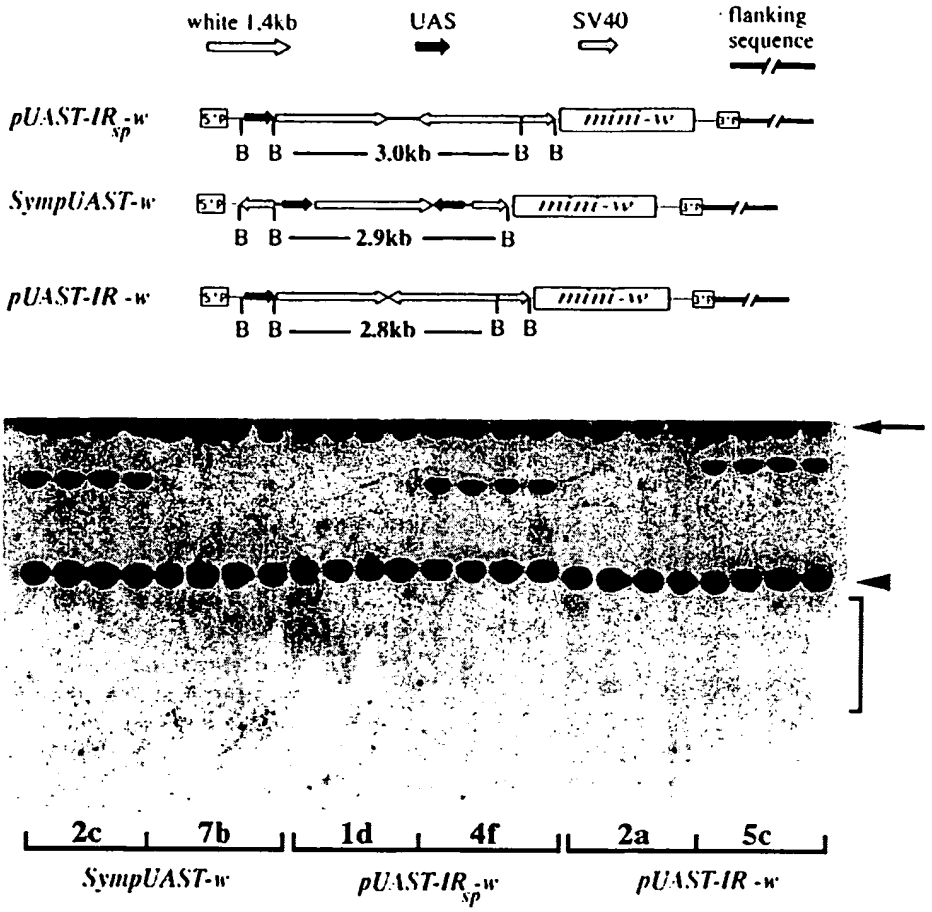

FIGURE 4.—Structural stability of the IR and symmetrically transcribed *w* transgenes examined by Southern blotting genomic analysis. Transgenic flies carrying either the *pUAST-IR-w*, *pUAST-IR$_{sp}$-w*, or *Sym-pUAST-w* transgene, all homozygous for the w^{1118} allele (a *white* partial deletion), were examined. Two independent lines (indicated at the bottom) were analyzed for each transgene. For each line, four samples of 5 μg of genomic DNA, each extracted from a pool of 30 adult flies, were digested with *Bam*HI (B) and analyzed using the 1.4-kb *w* cassette as probe. On the basis of their specific restriction patterns (shown at the top; note that the map is not precisely to scale), bands of 2.9, 3.0, and 2.8 kb (indicated by the arrowhead) were expected from the *Sym-pUAST-w*, *pUAST-IR-w$_{sp}$*, and *pUAST-IR-w* transgenes, respectively. A band of variable size (≥4.7 kb), whose length was specific for each line, derived from the mini-*w* reporter and the genomic sequences flanking the insertion site, while a third band of largest size (marked by the arrow) derived from the endogenous w^{1118} allele. Additional hybridizing bands, whose position is marked by the bracket, are noted in some of the *pUAST-IR-w* samples and might correspond to a truncated version of this transgene.

which derived from the transposon (see the restriction map in Figure 4), while a third, of largest size (marked by the arrow in Figure 4), derived from the endogenous w^{1118} allele. One of the transposon's bands was expected to be of 2.8, 2.9, or 3.0 kb, depending on the specific transgene (the position of these bands is marked by the arrowhead in Figure 4), while the second, derived from the mini-*w* reporter, was expected to be a different size (≥4.7 kb) in each independent line. In fact, one end of this fragment was located within the transposon, with the other being located in the flanking genomic sequence. Surprisingly, novel bands, whose intensity was less than that of single-copy sequences, were detected in some of the samples derived from each of the two *pUAST-IR-w* lines. These bands might presumably derive from rearrangements occurring at the *pUAST-IR-w* locus. With only one exception, these bands were <2.8 kb (the position of most of these additional bands is marked by the bracket in Figure 4). It is therefore plausible that they might involve large deletions in the region spanning the mini-*w* reporter or partial deletions occurring in the IR region. In that case, they might represent heterogeneous truncated forms of the transgene, readily accounting for the phenotypic heterogeneity of *pUAST-IR-w* silenced flies. In mice carrying perfect palindromic transgenes, rearrangement of the inverted repeat has been reported to occur frequently in either somatic or germline cells (COLLICK *et al.* 1996), being detectable in some cases in 15–56% of the transgenic progeny (AKGUN *et al.* 1997). Most frequently, these events produce small asymmetric deletions at the center of the palindrome that lead to a stabilized structure (COLLICK *et al.* 1996; AKGUN *et al.* 1997). Although we have not determined the precise nature of rearrangements occurring at the *pUAST-IR-w* transgenic locus, it is plausible that it might similarly undergo the same types of events described in mice.

Noticeably, no trace of rearrangements was found in flies carrying the *pUAST-IR$_{sp}$-w* transgene (Figure 4), nor were phenotypic mosaics observed among these transgenic individuals, suggesting that a spacing length of 200 bp can be sufficient to allow a stable mitotic inheritance of IR transposons. This observation is in good agreement with a recent report that indicated that the recombinogenic potential of long, perfect genomic IRs is strongly reduced by increasing the distance between the repeats (LOBACHEV *et al.* 2000).

Gal4-dependent gene silencing can be restricted to a localized body domain of the fly: To assess the general effectiveness of symmetrically transcribed transgenes and to test their ability to silence gene expression in specific body regions only, we attempted to interfere

with the expression of a Drosophila gene involved in an essential, ubiquitous metabolic process. For this purpose we chose *minifly* (*mfl*, also called *Nop60b*; see PHILLIPS *et al.* 1998), a gene involved in ribosome biogenesis whose defective mutations have been characterized in our laboratory (GIORDANO *et al.* 1999). *mfl* provides an ideal target for RNAi, given that *mfl*-defective alleles are characterized by quantitative alterations of the gene product. Functional analysis of these alleles, all due to *P*-element insertions outside the coding sequence, showed that the level of *mfl* transcription was critical, so that a dose-effect rule modulates the mutant phenotypes. In fact, strong loss-of-function mutations cause larval lethality, while partial loss-of-function alleles produce a viable hypomorphic phenotype characterized by pleiotropic defects, such as small body size; developmental delay; hatched abdominal cuticle; reduction in the number, length, and thickness of bristles; and reduced female fertility (GIORDANO *et al.* 1999).

To interfere with *mfl* expression we then prepared *Sym-pUAST-mfl*, a transgene allowing symmetrical transcription of a 1.7-kb segment derived from the *mfl* gene (see MATERIALS AND METHODS), and a second transgene, named *pUAST-IR$_{sp}$-mfl*, in which two inverted repeated copies of the same 1.7-kb *mfl* segment, spaced by 200 bp, were introduced into the *pUAST* vector. Both transgenes were then ubiquitously activated by the *Act5-Gal4* driver and the corresponding silenced flies were compared phenotypically. Ubiquitous activation of the *Sym-pUAST-mfl* transgene led to developmental delay and reduced female fertility, with a large percentage (>90%) of flies showing hatched abdominal cuticle and reduction in the number, length, and thickness of bristles, all features consistent with a partial loss-of-function *mfl* phenotype. In contrast, *pUAST-IR$_{sp}$-mfl/Act5-Gal4* heterozygotes showed late-larval or pupal lethality, indicating that in these flies *mfl* expression was reduced below the critical threshold. To disrupt the *mfl* activity only in selected cell types, we then crossed the *Sym-pUAST-mfl* and *pUAST-IR$_{sp}$-mfl* transgenic lines to *pnr-Gal4*, a strain expressing *Gal4* under the control of the promoter of the *pannier* (*pnr*) gene. As shown in Figure 5A, this promoter drives *Gal4* expression specifically along a mid-dorsal band (CALLEJA *et al.* 1996). Remarkably, *Sym-pUAST-mfl/pnr-Gal4* heterozygotes exhibited a strict localization of the defects typical of the *mfl* hypomorphic phenotype, showing a reduction in the number, length, and thickness of bristles only along the dorsal midline (Figure 5B). These traits, which reproduced with fidelity the phenotypic defects typical of the *mfl^1* allele within the *pnr* dorsal domain, indicated that *mfl* function was partially disrupted within this specific region only. This observation substantially confirms that symmetrically transcribed transgenes can be of general validity in inducing RNAi and that the silenced phenotypes are those expected for a partial loss-of-function of the target gene. As expected on the basis of the previous results, the phenotype of *pUAST-IR$_{sp}$-mfl/pnr-Gal4* silenced flies was instead more extreme, being characterized by a nearly total depletion of bristles within a body region that, although essentially centered along the dorsal midline, appeared to extend slightly beyond the *pnr* domain (Figure 5C, line *2c*). The phenotype of one of the two independent transformed lines analyzed was even more strongly affected, since the silenced flies were further characterized by the presence of a cleft in the middle of the thorax (Figure 5C, line *3a*). This cleft presumably results from a failure of the two halves of the thorax to fuse correctly at the dorsal midline during metamorphosis, suggesting the occurrence of localized cell death during the late larval or early pupal stages. As *mfl* is an essential gene, its restricted inhibition is indeed expected to cause cell death within the specific body region. In fact, apoptotic cell death has been described in the ovaries of females homozygous for a *mfl* partial loss-of-function mutation (GIORDANO *et al.* 1999).

Interestingly, the finding that both types of *mfl* silenced flies showed highly localized defects indicates that RNAi triggered by both types of transgenes is not substantially spread to the neighboring cells. This contrasts with that previously reported for worms (FIRE *et al.* 1998) and Plana-

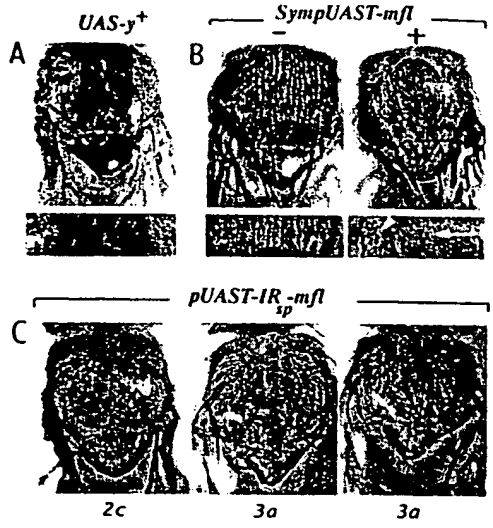

FIGURE 5.—Localized RNA interference of *mfl* expression. (A) Visualization of the body domain in which *Gal4* expression is restricted in the *pnr-Gal4* driver line. The *pnr* domain is marked by the y^+ dorsal band, due to the localized expression of a *UAS-y*$^+$ transgene in a fly of *y* mutant genotype that carried the *pnr-Gal4* activator transposon. (B) Phenotypes of females in which the *Sym-pUAST-mfl* transgene was either inactive (−) or activated by the *pnr-Gal4* driver (+). In these silenced flies, a reduction in the length and thickness of bristles occurs within the *Gal4* expression domain (marked by the arrow in the enlargement at bottom; the arrowhead marks the external region), reproducing the phenotypic defects typical of the *mfl* hypomorphic mutation only along the dorsal midline. (C) Phenotypes of *pUAST-IR$_{sp}$-mfl/pnr-Gal4* silenced females. The two independent transformed lines (*2c* and *3a*) analyzed were both characterized by absence of dorsal bristles within the *pnr* dorsal domain; in addition, occurrence of a cleft at the dorsal midline is observed in individuals of line *3a*.

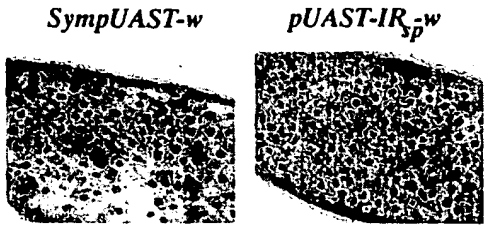

FIGURE 6.—Nuclear localization of transcripts derived by RNA interference vectors. *In situ* hybridization of a *w* antisense probe strongly labeled the nuclei of ovary follicle cells from both *Sym-pUAST-w* or *pUAST-IR-w$_{sp}$* silenced females. Identical distribution was observed with a *w* sense probe.

ria (SANCHEZ ALVARADO and NEWMARK 1999), where RNAi can spread from the site of interfering RNA application, and with the systemic spread of transgene-induced cosuppression states observed in plants (PALAUQUI *et al.* 1997; VOINNET and BAULCOMBE 1997). Although it is presently unclear whether this reflects a peculiar difference distinguishing the process of RNAi occurring in Drosophila from that of other organisms, it certainly indicates that, at least in flies, the *Gal4/UAS* binary system can productively be utilized to trigger RNAi in restricted body domains.

Nuclear localization of transgenic transcripts: We wished to investigate the possibility that the transcripts derived from symmetrically transcribed or IR transgenes might have any specific subcellular localization *in vivo*. Interestingly, *in situ* hybridization experiments revealed that both the sense and antisense transcripts accumulated at high levels within the nuclei of ovary follicle cells of flies silenced for both *w* (Figure 6) and *mfl* (data not shown), regardless of the specific type of construct they carried. While the *mfl* gene is expressed constitutively (GIORDANO *et al.* 1999), the *w* gene is inactive in follicle cells. This indicates that the transgenic transcripts accumulate within the nuclei regardless of the presence of the homologous target mRNA. Although further experiments are required to assess the functional significance, if any, of this unusual localization, it is possible that it may reflect the existence of a nuclear step of the RNAi process. To this regard, it is interesting to note that *in situ* experiments performed in *C. elegans* cells have similarly revealed the presence of a strong nuclear signal after RNAi (MONTGOMERY *et al.* 1998). More recently, the biochemistry of RNAi has been intensively investigated by using Drosophila embryo extracts (TUSCHL *et al.* 1999). The results obtained by several authors have indicated that short sense and antisense 21- to 22-nucleotide (nt) RNAs are produced from both dsRNA and the target mRNA during a two-step process (HAMMOND *et al.* 2000; KETTING and PLASTERK 2000; ZAMORE *et al.* 2000). In a first step, a RNaseIII-type protein, called Dicer-1, degrades dsRNA into ~21-nt dsRNA intermediate active forms [short interfering RNAs (siRNAs)], forming a siRNA-Dicer complex (BERNSTEIN *et al.* 2001), while in a second step this complex might recruit additional proteins, recognizing and cleaving the target RNA. Although it is well established that the RNAi process leads to a substantial loss of the cytoplasmic target mRNA, it cannot presently be excluded that one of these steps might, at least in part, take place in the nucleus. Compartmentalization of separate dsRNA-processing pathways in the nucleus and cytoplasm has in fact been hypothesized (MATZKE *et al.* 2001). Moreover, it has recently been suggested that the active degradation complex might cleave the target RNA at the nuclear pores, as it exits the nuclear compartment (WATERHOUSE *et al.* 2001). Finally, a candidate Dicer homolog in Arabidopsis has been noted to contain two bipartite nuclear localization signals (JACOBSEN *et al.* 1999; BERNSTEIN *et al.* 2001).

CONCLUSIONS

Our results show that symmetrically transcribed transgenes, activated by the *Gal4/UAS*-inducible expression system, can successfully be utilized to trigger RNAi in Drosophila and that their use can be particularly valuable when partial loss-of-function mutant phenotypes are desirable. Symmetrical transcription of transgenes was reported to induce effective RNAi also in Trypanosoma (WANG *et al.* 2000), indicating that this approach might be of general validity and possibly be extended to other organisms. Our data also provide direct evidence that endogenous dsRNA formed by simultaneous transcription of sense-antisense strands can actually represent a cellular regulatory signal able to trigger a genetic repressed state. Intriguingly, *in vivo* sense-antisense transcription has been invoked as a possible mechanism for the regulation of several genes (LIPMAN 1997; EDDY 1999), and many biological phenomena, as, for example, X chromosome inactivation or imprinted gene expression in mammals, are known to involve transcription of both strands at specific loci (see HEARD *et al.* 1999; SLEUTELS *et al.* 2000 for reviews). In Drosophila, both strands of the *Suppressor of Stellate* [*Su(Ste)*] locus are transcribed, and the production of *Su(Ste)* dsRNA has been implicated in the silencing of the paralogous *Stellate* (*Ste*) gene (ARAVIN *et al.* 2001). Given the strong silencing effect exerted by dsRNA molecules in a large variety of organisms, it is possible that symmetrical transcription of endogenous genomic sequences might play a wider role in the regulation of gene expression than previously suspected.

Concerning the functional analysis of the Drosophila genome, our results might also furnish a useful clue toward a better comprehension of the mechanisms accounting for the unexpected high efficiency displayed by a recently developed gene search (GS) mutagenesis approach. This method makes use of *P*-element vectors containing copies of the *UAS* sequence oriented to direct outward transcription bidirectionally (TOBA *et al.*

1999), with the principal aim of eliciting over- or ectopic expression of the target gene. This would lead to the collection of gain-of-function mutations, favoring the identification of genes that are not uncovered by loss-of-function phenotypes. The efficiency of the GS method, much higher than that of classical *P*-element mutagenesis schemes, was unexpected and so far substantially unexplained (TOBA *et al.* 1999). We suggest that its high efficiency is based in part on the potential ability of this vector to interfere with the expression of the target gene by producing dsRNA. In fact, the capability of triggering outward transcription bidirectionally makes the transposon able to induce either loss- or gain-of-function mutations, depending on the transcriptional polarity of the target gene. On this basis, we surmise that, despite the expectation, a significant portion of the mutations isolated by this method might be loss-of-function instead of the expected gain-of-function alleles.

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